Activation of c-Jun amino-terminal kinase is required for retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells

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Abstract P19 embryonal carcinoma cells are known to differentiate into neurons and glia when treated with relatively high concentrations (>100 nM) of retinoic acid (RA). Concomitant with this RA-induced neural differentiation, we observed an activation of the c-Jun amino-terminal kinase (JNK). JNK was required for the RA-induced neural differentiation, because dominant-negative JNK blocked the differentiation. Studies using protein phosphatase inhibitors and protein kinase inhibitors suggested that both okadaic acid-sensitive protein phosphatase(s) and protein kinase C participate in the RA-induced activation of JNK. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: P19 embryonal carcinoma cell; Retinoic acid; Neural differentiation; c-Jun amino-terminal kinase; Okadaic acid-sensitive protein phosphatase; Protein kinase C

1. Introduction

P19 cells are a murine embryonal carcinoma cell line that have been used extensively as a model system for studying early embryonic development and differentiation. These pluripotent cells resemble the inner cell mass of early embryos and can differentiate into endoderm, mesoderm or ectoderm depending on the culture conditions [1–3]. Treatment of aggregated P19 cells with low concentrations (10 nM) of retinoic acid (RA) leads to differentiation to endodermal and mesodermal derivatives [1–3]. At higher concentrations (>100 nM) of RA, P19 cells can be stimulated to form neurons and glia [1–3].

Studies on signaling systems responsible for the RA-induced differentiation of P19 cells have indicated that the c-Jun amino-terminal kinase (JNK) signaling pathway plays important roles [4,5]. Expression of a dominant-negative form of JNK (dnJNK) blocked RA-induced endodermal differentiation [4]. In addition, stable expression of c-Jun in P19 cells have been found to induce endodermal and mesodermal differentiation [5]. These observations indicate that the activity of JNK is required for either endodermal or mesodermal differentiation of P19 cells. However, it is not known whether JNK is a major player in the neural differentiation of P19 cells, which is induced by high concentrations of RA.

Here, we present evidence that the activation of JNK through a pathway which is sensitive to both protein phosphatase 1 and 2A inhibitors and protein kinase C (PKC) inhibitors is required for the RA-induced neural differentiation of P19 cells.

2. Materials and methods

2.1. Materials

The restriction enzymes and other modifying enzymes for DNA manipulation were purchased from Takara (Kyoto, Japan). [γ-^{32}P]ATP (3000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA, USA). The anti-mouse neurofilament-L (NF-L) and anti-Flag antibodies were from Zymed Labs (San Francisco, CA, USA). The anti-SAPK/JNK antibodies were from Santa Cruz (Santa Cruz, CA, USA). The anti-phospho-SAPK/JNK antibody and the SAPK/JNK assay kit were purchased from New England Biolabs (Beverly, MA, USA). Anti-mouse and anti-rabbit IgG-alkaline phosphatase conjugates were from Promega (Madison, WI, USA). PKC inhibitors (calphostin C and chelerythrine chloride) were from Biomol (Plymouth Meeting, USA). All other reagents used were from Wako Pure Chemical (Osaka, Japan).

2.2. Methods

2.2.1. Preparation of expression plasmids. A dominant-negative point mutant of JNK1 was prepared by substitution of phosphorylation sites (Thr183 and Tyr185) of wild-type (wt)JNK1 (a gift from Dr. Michael Karin, University of California in San Diego, CA, USA) with Ala and Phe, respectively, using a site-directed mutagenesis kit (Takara). The resulting mutant DNA was designated by JNK1(APF). A Flag-tag was inserted into the amino-terminal end of JNK1 and JNK1(APF) using polymerase chain reaction (PCR). Both wtJNK and mutant JNK1 were subcloned into the pCX expression vector (provided by Dr. Taeko Miyagi, Miyagi Cancer Centre, Japan) and named pCX-Flag-JNK1 and pCK-Flag-JNK1(APF), respectively.

2.2.2. Cell culture and transfection. P19 EC cells were obtained from the American Tissue Culture Center (Rockville, USA) and cultured in bicarbonate-buffered α-modified Eagle's medium supplemented with 10% (v/v) fetal calf serum under a humidified atmosphere with 5% (v/v) CO_2 in air at 37°C. To induce neural differentiation, the cells were cultured on bacterial-grade dishes to form aggregates (aggregation culture) for 4 days in the presence of 1 μM all-*trans* RA, then replated on tissue culture-grade dishes and cultured (monolayer culture) for 3 more days without RA [6]. For the treatment with inhibitors, the cells were preincubated with okadaic acid (OA, 10 nM), tautomycin (50 nM), calphostin C (0.25 μM) or chelerythrine chloride (3 μM) for 30 min at 37°C before RA was added to the medium. These protein phosphatase inhibitors and PKC inhibitors were present in the culture medium throughout the 7-day differentia-

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Abbreviations: RA, retinoic acid; JNK, c-Jun amino-terminal kinase; PKC, protein kinase C; NF-L, neurofilament-L; PCR, polymerase chain reaction; OA, okadaic acid

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tion process. For stable transfection, P19 cells were co-transfected with pBABE, encoding the puromycin resistant gene, and pCX-Flag-JNK1 or pCX-Flag-JNK1(APF) using the Lipofectamine method (Gibco BRL, Rockville, USA). Puromycin-resistant colonies were isolated after selection.

2.2.3. Preparation of cell extracts and immunoblot analysis. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in a lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% (v/v) Triton X-100, 50 mM NaF, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). Each suspension was centrifuged at $10\,000\times g$ for 10 min and the resulting supernatant was used as cell extracts. Aliquots of the extracts (20 μg protein) were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), then electrotransferred onto PVDF transfer membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and immunostained with the indicated antibodies. The immunoreactivity was detected with chemiluminescent CDS-StarTM (Boehringer Mannheim, Mannheim, Germany). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.4. Protein kinase assay. Determination of JNK activity in the cell extracts was performed by immune complex kinase assay using GST-fused c-Jun as the substrate for JNK, as described previously [7]. Aliquots of the cell extracts (500 μg proteins) were immunoprecipitated with anti-JNK and protein A-agarose beads. The immunoprecipitates were resuspended in 30 μl of kinase reaction buffer A (25 mM Tris–HCl, pH 7.4, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT and 20 mM $MgCl_2$) containing 3 μg of GST-c-Jun, 50 μM ATP and 5 μCi [γ-^{32}P]ATP, and incubated for 15 min at 30°C. The reactions were terminated by addition of the SDS sample buffer, resolved on SDS–PAGE and the incorporation of ^{32}P into the substrate proteins was visualized and quantitated with the BAS 2000 imaging analyzer (Fuji, Tokyo, Japan).

2.2.5. Detection of apoptosis and immunostaining of cells. The cells on day 5.5 of the neural differentiation process were fixed in 4% (w/v)

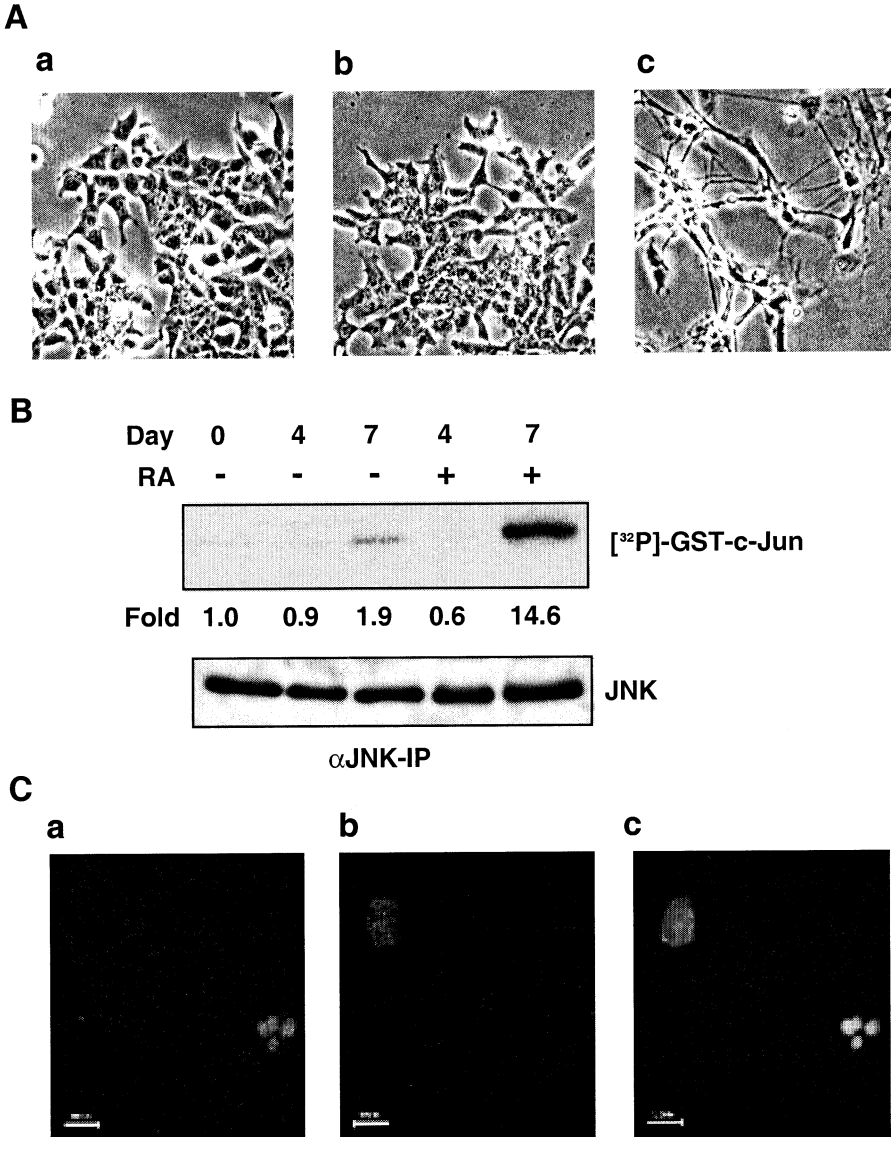

Fig. 1. Activities of JNK in the course of RA-induced neural differentiation of P19 cells. A: Photomicrographs showing RA-induced neurite outgrowth of P19 cells are depicted. P19 cells (day 0, a) were cultured in the absence (b) or in the presence (c) of 1 μM RA on bacterial grade culture dishes for 4 days, then replated onto the tissue culture grade dishes and cultured for another 3 days in the absence of RA. B: P19 cells were treated as described in the legend of A and harvested at day 0, 4 and 7. Aliquots of cell extracts (500 μg proteins) were immunoprecipitated with anti-JNK antibody and the kinase activity was determined by the incorporation of ^{32}P into GST-c-Jun (upper panel). The protein level of JNK was monitored by immunoblot analysis using anti-JNK antibody (lower panel). The results represent one of three independent experiments. C: The cells on day 5.5 of the neural differentiation process were stained with an in situ apoptosis detection kit (a). The fragmented DNA of a TUNNEL-positive cell is shown. The cells were also stained with anti-phospho-JNK antibody (b) or Hoechst 33258 (c).

paraformaldehyde in PBS for 15 min. The fixed cells were then permeabilized with 0.1% (v/v) Triton X-100. Apoptotic cells were stained with an in situ apoptosis detection kit (Takara) according to the manufacturer's instructions. Subsequently, the cells were incubated with anti-phospho-JNK antibody (New England Biolabs) for 1 h, then immunodetected with Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, PA, USA).

3. Results

3.1. JNK is activated in the course of RA-induced neural differentiation of P19 cells

The morphological changes during the course of RA-dependent neural differentiation of P19 EC cells are shown in Fig.

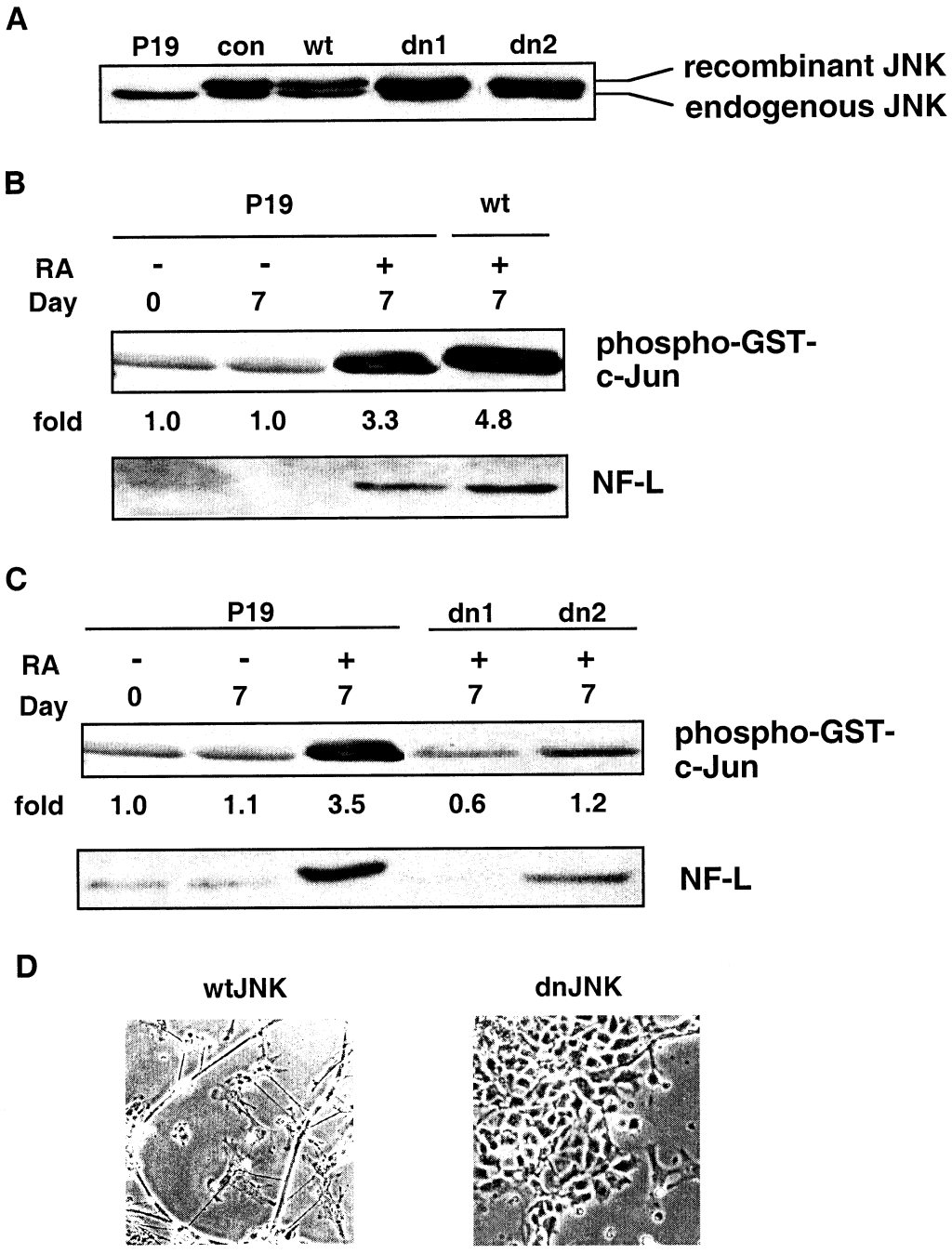

Fig. 2. Effect of expression of wtJNK or dominant-negative JNK on the RA-induced neural differentiation of P19 cells. P19 cells were co-transfected with pBABE, encoding puromycin-resistant gene, and the expression plasmid of pCX-Flag-JNK1 or pCX-Flag-JNK1(APF) using the Lipofectamine method. Puromycin-resistant colonies of cells were isolated and the expression levels of the endogenous and ectopic forms of JNK were examined by immunoblot analysis using anti-JNK antibody (A). The extracts of P19 cells transiently transfected with pCX-Flag-JNK1 plasmid was used as the positive control (con). The transfectants of the wild-type (B) and the mutant forms (C) of JNK were treated with RA as described in the legend of Fig. 1 and harvested at day 0 or day 7. The JNK activities of the transfectants were determined by phosphorylation of GST-c-Jun using a specific anti-phospho-c-Jun antibody (SAPK/JNK assay kit) (B and C, upper panels). The expression of NF-L was examined by immunoblotting with the anti-NF-L antibody (B and C, lower panels). The results shown in B and C represent one of three independent experiments. The microscopic appearance of the cells transfected with pCX-Flag-JNK1 (D, left panel) or pCX-Flag-JNK1(APF) (D, right panel) on day 7 is shown.

1A. While these cells retained a fibroblast-like appearance for 7 days in culture without added RA (Fig. 1A-b), neurite outgrowth was observed on day 7 of the RA-induced neural differentiation process (Fig. 1A-c). No change in JNK activity was observed in the cell extracts on day 4 of the 7-day differentiation process, whether or not RA had been added (Fig. 1B, upper panel). By day 7 without RA there was a slight increase in JNK kinase activity. In contrast, RA treatment produced a large increase in JNK kinase activity starting at day 5, measured by ^{32}P incorporation into GST-c-Jun. In the experiment shown, there was a 7.7-fold increase in JNK activity at day 7, compared to day 7 control without added RA (1.9 vs. 14.6) (Fig. 1B, upper panel). In parallel with the increased activity, the phosphorylation of JNK detecting by anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody, was enhanced on day 7 of the RA-induced differentiation process

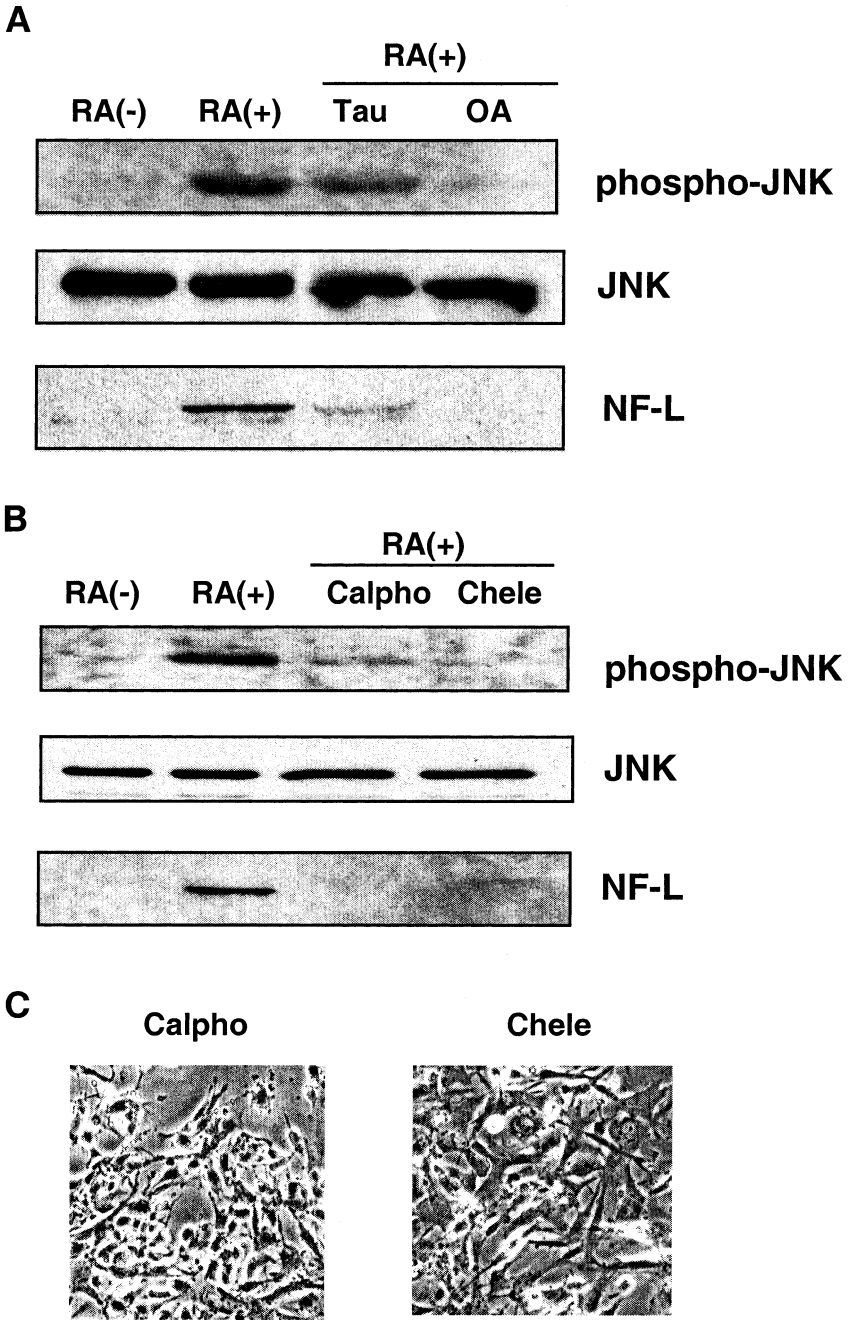

Fig. 3. Effects of protein phosphatase inhibitors and PKC inhibitors on the phosphorylation of JNK and RA-induced neural differentiation of P19 cells. A, B: P19 cells were treated with or without 10 nM OA or 50 nM tautomycin (A) or 0.25 μM calphostin C or 3 μM chelerythrine chloride (B) throughout the 7-day differentiation process. Untreated cells were used as a control. On the 7th day, the cells were harvested and lysed. The phosphorylation levels of JNK were examined by immunoblot analysis using anti-phospho-JNK antibody (top panels). The protein levels of JNK were monitored by immunoblot analysis using anti-JNK antibody (middle panels). The expression of NF-L was determined by immunoblot analysis using anti-NF-L antibody (bottom panels). C: The microscopic appearance of the cells on day 7 in the presence of calphostin C (left panel) or chelerythrine chloride (right panel) is shown.

(Fig. 3A,B), with little change in the JNK protein levels over the course of differentiation (Fig. 1B, lower panel). Immunostaining of the cells at day 5.5 of the differentiation process with anti-phospho-JNK antibody indicated that active JNK is localized in the nuclei of TUNNEL-negative cells (Fig. 1C). These results indicate that selective activation of JNK by phosphorylation takes place in the course of RA-induced neural differentiation of P19 cells.

3.2. dnJNK suppresses the RA-induced neural differentiation of P19 cells

We established P19 cell lines stably expressing wtJNK and P19 cell lines stably expressing dnJNK (T183A/Y185F) (Fig. 2A). As shown in Fig. 2B (upper panel), in non-transfected P19 cells RA-induced neural differentiation corresponded to a 3.3-fold increase in JNK activity measured by anti-phospho-c-Jun immunoblotting. By comparison, P19 cells stably expressing wtJNK showed a 4.8-fold increase in JNK activity after 7 days of RA-induced neural differentiation. No change in JNK activity or fibroblast phenotype was observed in the cell line expressing the wtJNK without added RA (data not shown). Concomitant with the enhanced activity of JNK, the expression level of NF-L (Fig. 2B, lower panel) and the neurite outgrowth (Fig. 2D, left panel) were also enhanced by the ectopic expression of wtJNK. In contrast, the cell line expressing the dnJNK (dn1, dn2) did not show increased JNK activity relative to the control cells at day 7 of the RA-induced differentiation process (Fig. 2C, upper panel). In addition, both NF-L expression (Fig. 2C, lower panel) and the neurite outgrowth (Fig. 2D, right panel) were substantially suppressed in the cells expressing the dnJNK, compared to the control cells. These results indicate that activation of JNK is required for the RA-induced neural differentiation of P19 cells. This, plus other evidence, also suggest that the activation of JNK is commonly required for the differentiation of P19 cells into derivatives of all three germ layers – endoderm, mesoderm and ectoderm.

3.3. Treatment with protein phosphatase inhibitors or PKC inhibitors suppresses phosphorylation of JNK in the course of RA-induced neural differentiation of P19 cells

We have already reported that RA-induced differentiation of P19 cells was inhibited by the presence of the protein phosphatase inhibitors OA or tautomycin in the culture medium throughout the differentiation process [6]. Therefore, we studied whether the treatment with OA or tautomycin would affect the phosphorylation of JNK during the course of differentiation. As shown in Fig. 3A (top panel), the differentiation-dependent increase in the phosphorylation of JNK was substantially suppressed by the presence of OA or tautomycin in the culture medium. In parallel with the suppression of JNK phosphorylation, the differentiation-dependent increase in the expression of NF-L was also inhibited by OA or tautomycin, as previously observed (Fig. 3A, bottom panel) [6].

It has been reported that overexpression of epsilon-PKC markedly increased nerve growth factor (NGF)-induced neurite outgrowth of PC12 cells [8]. PKC also participates in the activation of JNK [9–13]. Addition of two membrane-permeable inhibitors of PKC, calphostin C and chelerythrine chloride, suppressed RA-induced JNK phosphorylation (Fig. 3B, top panel). In addition, these inhibitors prevented induction of NF-L (Fig. 3B, bottom panel) and neurite outgrowth in response to RA treatment (Fig. 3C). Neither of these PKC inhibitors had cytotoxic effects at the concentrations employed in the experiments (data not shown). These results suggested that both OA-sensitive protein phosphatase(s) and PKC participate in the activation of JNK in the course of RA-induced P19 cell neural differentiation.

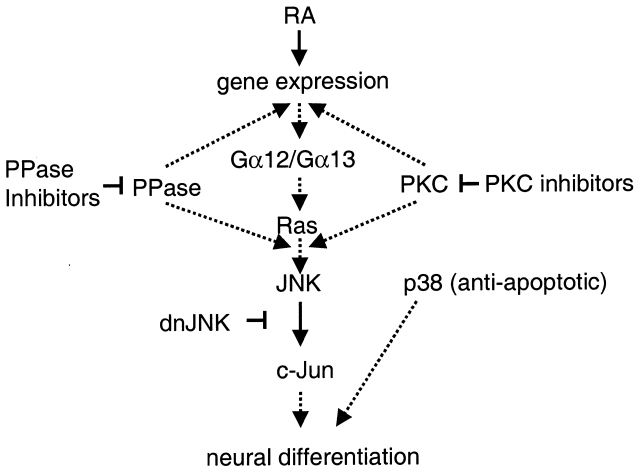

Fig. 4. Schematic model of regulation of RA-induced neural differentiation of P19 cells. PPase, protein phosphatase.

4. Discussion

In this study, we demonstrated that JNK is activated, with little change in the JNK protein levels, during the course of the RA-induced neural differentiation of P19 cells. In addition, experiments using the ectopic expression of the wtJNK or dnJNK in P19 cells indicated for the first time that the activation of JNK is required for neural differentiation (Fig. 2). In this context, the participation of JNK in endodermal differentiation of P19 cells using a distinct differentiation-inducing system had been reported by Jho et al. [4]. Signaling systems related to neural differentiation have been well documented in NGF-induced neural differentiation of PC12 cells, a rat pheochromocytoma cell line [14–17]. Studies have revealed that the activation of JNK and p38 kinase occurs in the differentiation of PC12 cells [15–17]. In this context, Okamoto et al. have recently reported that phosphorylation of p38 kinase was enhanced during induction of neural differentiation by RA [18]. Their results also indicated that the activation of p38 kinase plays an anti-apoptotic role during neural differentiation. These results suggest that both JNK and p38 play important roles in RA-induced P19 cell neural differentiation.

In addition, the G-proteins Gα12 and Gα13 have been implicated in the activation of JNK through a Ras-dependent signal transduction pathway [19]. The expression of Gα12 and Gα13 also was enhanced and essential to stimulation of endodermal and neuroectodermal differentiation of P19 cells by RA [20]. Therefore, Gα12 and Gα13 may participate in the JNK activation which takes place concomitant with the neural differentiation of P19 cells. However, the molecular mechanisms of the Gα12/Gα13 activation and the connection between Gα12/Gα13 and JNK in the course of the P19 cell differentiation have not been clarified. In this study, the two distinct PKC inhibitors of protein phosphatase (OA and tau-

tomycin) and the two distinct inhibitors (calphostin C and chelerythrine chloride) suppressed the neurite outgrowth and the NF-L expression as well as the RA-induced enhanced phosphorylation of JNK. Therefore, we suggest that both OA-sensitive protein phosphatase(s) and PKC participate in the differentiation-induced activation of JNK. The short-term incubation of neurally differentiated cells with OA or PKC inhibitors did not affect the JNK phosphorylation level (data not shown). Therefore, it is likely that both OA-sensitive protein phosphatase(s) and PKC stimulate JNK phosphorylation through an indirect mechanism, possibly by regulating the expression levels or activities of proteins which are involved in the activation mechanism of Gα12 and Gα13 or in the signaling pathway between Gα12/Gα13 and JNK (Fig. 4).

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